CHAPTER 3

PHASE III: FINAL SOURCE IDENTIFICATION REPORT

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1. Introduction

The San Juan Creek (SJC) Watershed Bacteriological Study was conducted to characterize the fecal indicator bacteriology of the watershed and to determine the sources of bacterial pollution using a combination of bacteriologic monitoring surveys and source tracking methods. The potential sources of fecal contamination identified in the SJC watershed include humans, sewage, storm drains, waterfowl, pets, horses and wild animals. During Phase I, water samples were collected from 36 sites in the watershed and bacterial densities were determined for total and fecal coliforms and *Enterococcus*. High levels of fecal indicator bacteria were consistently found in storm drains while moderate levels were detected in all the creek samples. However, no single source of bacterial pollution was identified based on the bacteriological monitoring results. During Phase II, five sites representative of different areas of the watershed were sampled for total and fecal coliforms, *Enterococcus* and *E. coli*. Bacterial levels were used to determine temporal and geographical differences in pollution and to identify the potential sources of contamination.

In addition to conducting a monitoring study, bacterial isolates were obtained from a variety of fecal and water samples for source tracking analysis to be conducted during Phases III and IV. The objectives were to identify specific sources or host species of fecal indicator bacteria using two different types of source tracking methods, Antibiotic Resistance Analysis (ARA) and ribotyping. Dr. Valerie Harwood of the University of South Florida (USF), in Tampa, Florida conducted the ARA analysis and Dr. George Lukasik of Biological Consulting Services (BSC) in Gainesville, Florida performed the ribotyping testing. In Phase III, large numbers of bacterial isolates obtained from animal and human fecal samples were used to create E. coli and Enterococcus databases or "libraries" required for ARA and ribotyping testing. Once the libraries were constructed using isolates from known sources, the accuracy and reproducibility of the methods were evaluated using unknown isolates. The purpose of Phase IV was to determine the sources of the watershed bacterial isolates using the ARA and ribotyping methods. However, Phase IV was not completed upon determination that the accuracy for both ARA and ribotyping in identifying specific host species for E. coli and Enterococcus isolates from the SJC Watershed were not sufficient.

2. Experimental Design/Sampling Plan

A. Sample Collection and Preparation

Environmental Samples

During Phase II, Orange County Public Health Laboratory (OCPHL) staff collected and analyzed 68 water samples for total and fecal coliform, *Enterococcus*, and *E. coli*. *E. coli* and *Enterococcus* bacterial strains were isolated from the water samples collected from 5 sites (Table 1). The water samples were tested for total coliforms, fecal coliforms, *Enterococcus* and *E. coli* using the membrane filtration methods (SM9222A & B, USEPA method 1600, USEPA modified *E. coli* Method (1998), respectively) described in Standard Methods for the Examination of Water and Wastewater, 20th edition and USEPA Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and *Escherichia coli*. A large number of *E. coli* and Enterococci isolates (1,820 and 1,850, respectively) were frozen for source tracking analysis to be conducted during Phase IV, upon completion of ARA and ribotyping library analysis and method accuracy determination.

Library Samples

OCPHL collected human and animal fecal samples as well as sewage samples to obtain strains of E. coli and Enterococcus bacteria used to create the ARA and ribotyping libraries (Table 2). A total of 675 fecal samples (85 cat, 103 dog, 188 seagull, 109 horse, 190 human) were collected, most of which were used for both E. coli and Enterococcus testing. Sewage effluent (treated sewage) and influent (before treatment) samples were collected by South Orange County Wastewater Authority. Human fecal samples were obtained from Mission Hospital Regional Medical Center and San Clemente Hospital and Medical Center, both located within the San Juan Watershed. Cat and dog fecal samples were acquired from veterinary clinics also located near the study area. Seagull droppings were collected by OCPHL from the coastline of Doheny Beach. The fecal bacteria were isolated using CHROMagar ECC (CECC) (Hardy Diagnostics) and Enterococcosel (BBL) medias for isolation of E. coli and Enterococcus, respectively. Sewage samples were also processed using the membrane filtration method. The membranes were placed onto CHROMagar ECC (CECC) and m-EI (modified Enterococcus) (USPEPA Method 1600) medias for isolation of E. coli and Enterococcus, respectively. Up to five isolates each of E. coli and Enterococcus were obtained per fecal source and 10 isolates per sewage source, resulting in over 7,000 isolates. Both E. coli and Enterococcus isolates from the fecal and sewage samples were sent to the USF for ARA testing; only E. coli isolates were sent to BSC for ribotyping. The E. coli isolates tested for ribotyping were also a subset of the isolates tested by ARA.

B. Library Preparation

Both ARA and ribotyping require constructing large databases or libraries of isolate patterns based on the antibiotic resistance patterns (ARPs) or ribotypes of bacteria from known species before they can be used to identify bacteria as being human or animal-derived. Fecal sources used to create the San Juan Watershed libraries included cat, dog, horse, human, seagull, sewage influent and effluent. ARPs of bacterial isolates from these sources were determined using a battery of antibiotics at various concentrations. The ARPs were analyzed using discriminant analysis (DA) and isolates were classified according to the most likely host species source. The robustness of the library was evaluated by performing a holdout analysis. Isolates from various known sources were "held out" of the library, and were analyzed as if they were unknowns. The internal accuracy of the ARA library was measured by the average rate of correct classification (ARCC). The ARCC is the sum of the correct classifications for all source categories divided by the total number of strains in the database and expressed as a percentage.

The ribotyping library was created using ribotype (RT) profile or patterns of *E. coli* restriction fragments that were statistically analyzed for similarities and placed into "ribogroups". The percent similarity of RTs was determined using Jackknife analysis (Bionumerics software). The principle of the Jackknife method is to take out one entry or isolate from the list, and to classify it based on the maximum similarities with each group, i.e., the group with entries most similar to the entry being identified, without including the entry itself.

C. Technique Accuracy and Reproducibility Determination Using Proficiency Samples

Accuracy Testing Using Proficiency Samples

The accuracy and validity of the discriminatory function of the ARA and ribotyping methods was evaluated by comparing known *E. coli* and *Enterococcus* isolate profiles to the library profiles. *E. coli* and *Enterococcus* isolates (n=97) from known fecal and sewage samples were sent to USF as "blind" (source not identified) or proficiency samples to determine the efficiency of ARA in accurately determining the source(s) of bacterial isolates. These bacterial isolates were from samples collected concurrently with the samples used to create the libraries but kept frozen until the libraries were completed. The purpose for using isolates from known fecal sources that were not included in the library was to mimic the analysis of unknown environmental samples, while retaining the capability of judging the accuracy of ARA were also tested by BSC using ribotyping so that the accuracy of the methods could be compared directly.

Reproducibility Testing

The purpose of the reproducibility testing was to determine whether the ARA and ribotyping methods could produce the same results in terms of classifying isolates into source categories when the testing was repeated at least 3 times using the same set of samples. Sub-sets of bacterial isolates from the proficiency samples were used to test the reproducibility of ARA and ribotyping. The same set of *E. coli* proficiency isolates was used to test the reproducibility of both methods.

The ARA reproducibility study was designed to determine the consistency of repeated measures of the antibiotic resistance patterns of a selected group of *E. coli* and *Enterococcus* isolates over time. Twenty each of *E. coli* and *Enterococcus* isolates were subjected to ARA once a week for 3 weeks. Three replicate measurements of the ARP of each isolate were obtained each week. Therefore, a total of 9 ARP measurements for each isolate were conducted (3 per week for 3 weeks).

The ribotyping reproducibility study was conducted by sub-culturing each of the 20 *E. coli* proficiency isolates in triplicate and ribotyping the samples 3 different times.

3. Data Analysis

A major component of bacterial source identification involves analyzing the different bacterial patterns using several statistical techniques. In this study, the ARA library and accuracy testing was analyzed by discriminant analysis, SAS 8.0 (SAS Institute, Cary, NC). ARA reproducibility testing and ribotyping library and accuracy determination was analyzed using the Jackknife discrimination and Pearson clustering statistical programs (Bionumeric Software, Applied Maths, Austin TX). Regardless of the type of analysis used, the efficiency of the ARA and ribotyping methods to classify known isolates into correct source groups is measured by the ARCC and rate of correct prediction (RCP). The ARCC is the sum of the correct classifications for all source categories divided by the total number of strains in the database expressed as a percentage. RCP is the percentage of isolates correctly predicted divided by the total classified for each species. Unlike other published calculations in source tracking studies, the RCP accounts for both the correct classification rate and the rate of misclassification in each source category. The higher the RCP, the more accurate the classification of isolates into a given source category.

Determination of the library accuracy for ARA differed from ribotyping in terms of the number of source groups used. Whereas the accuracy of the ribotyping library was based on the ability to correctly classify an isolate into 1 of 7 source

categories, the accuracy of the ARA library was based on using 6 categories. The 7 categories were as follows: cat, dog, horse, seagull, human, sewage influent and sewage effluent. For ARA analysis, the sewage influent and effluent results were combined into a single "sewage" category. Correct classification rates generally increase with decreasing number of source categories, as long as isolates are being grouped into valid categories.

Since *E. coli* or Enterococci isolates from sewage influent or effluent samples could potentially be classified into categories other than human, the library and proficiency results were analyzed with and without including sewage as a category. In this study, the "human" category refers to clinical isolates from human subjects.

4. Results

Various source tracking techniques have recently been used to identify sources of fecal pollution in source water, however the accuracy or robustness of these methods has not been rigorously tested in the field. In previous studies, the accuracy of ARA methods was evaluated based on how well isolates within the library or database were classified or "self-crossed" (Wiggins, et al., 1996; Harwood et al., 2000). The efficiency of the library was based on the average correct classification rates for discriminating sources. However, additional validation of the library accuracy and reproducibility was not tested using proficiency or "blind samples". Thus, in this study, the accuracy of ARA and ribotyping for identifying specific sources of *E. coli* and *Enterococcus* isolates was also evaluated using a proficiency panel comprised of 100 bacterial isolates from known source species. The internal accuracy of the library as well as the accuracy and reproducibility determination based on the proficiency panels was evaluated for both ARA and ribotyping.

A. Antibiotic Resistance Analysis

Internal Accuracy of the ARA Library

The internal accuracy of the ARA libraries for *E. coli* and *Enterococcus* libraries is shown in Table 3. The source of the fecal isolates is listed in the first column of the classification table and the assigned classifications are listed in the top row. The ARCC for *E. coli* ARA library was 43.6% based on an average correct classification of 1,517 of a total of 3,477 isolates. The RCPs ranged from 26.9% for isolates from dogs to 63.6% for sewage isolates.

The ARCC for *Enterococcus* library was 47.7% based on correctly classifying 1,746 of a total of 3,657 isolates. The RCPs ranged from 25.7% for cats to

66.7% for sewage. For both fecal indicator ARA libraries, the sewage isolates had the highest rate of correct prediction for *E. coli* and *Enterococcus*.

Accuracy of ARA Based on Proficiency Testing

Ninety-seven *E. coli* and 99 *Enterococcus* isolates from 7 fecal sources were tested as "blind" samples. The source of the isolates was unknown to the USF laboratory performing the ARA, but known to OCPHL. The analysis of correct classification is presented in Table 4. The highlighted values show the number and percentage of isolates that were identified to the assigned group. Overall, the ARCC of the *E. coli* isolates (based on testing proficiency samples) was 28.9% as compared to 43.6% for the library. The RCPs ranged from 9.1% to 100%, however, in this case, the 100% RCP result was a statistical anomaly since only 1 human *E. coli* isolate was correctly classified while 15 human isolates were misclassified. The ARCC for human *E. coli* isolates was 6.3% as compared to 39.3% for the library.

As for *Enterococcus* isolates, the ARCC of the proficiency samples was 45.5%, which reflects the library ARCC of 47.7%. Sewage and horse isolates had the highest classification rates at 85.7% and 78.6%, respectively. However, none of the 16 human isolates were classified as human; 7 isolates were misclassified as cat and 4 were classified as sewage.

Reproducibility of ARA Based on Proficiency Testing

After the accuracy testing was conducted, a subset of the 97 proficiency samples was tested to assess method reproducibility. Twenty isolates of *E. coli* and *Enterococcus* were subjected to ARA on 3 different days. Three replicates were processed per day for a total of 9 results per isolate. Table 5 lists the proficiency results (Predicted Source, Trial 1) for comparison with the reproducibility results (Predicated Source Reproducibility Trials). The reproducibility results were also analyzed without sewage (data not shown). Of the 20 *E. coli* isolates tested, 3 isolates agreed for all 9 trials, 1 of which was identified to the correct source. Ten results agreed at least 6 out of 9 times, but only 2 were correct as to source. As for *Enterococcus*, only 1 of 20 isolates was correctly identified for all 9 trials (Table 6).

B. Ribotyping

Internal Accuracy of the Ribotyping Library

The *E. coli* ribotyping library was constructed based on the ribotype profile of 997 isolates that were also included in the ARA database. The proficiency of the library is presented in Table 7 as the "Maximum Similarity Jackknife Analysis of *E. coli* Ribotype Profiles". The source of the fecal isolates is listed in the first column of the classification table and the assigned classifications or categories

are listed in the top row. The range of percentage of maximum similarity ranged from 33.6% for effluent to 82.4% for horses. The ARCC for human isolates was 75.5%. Overall, the ARCC for *E. coli* using 7 sources was 63.8%.

Accuracy of Ribotyping Based on Proficiency Testing

The same set of 97 "blind" *E. coli* isolates analyzed for ARA accuracy testing was also used to determine the accuracy of the ribotyping method. Overall, the ARCC was 26.8% (ranging from 7.1% for sewage to 62.5% for human isolates) (Table 8). The ARCC did not change significantly when the results were also analyzed without including the sewage category (29.0% ARCC, data not shown). Based on testing the *E. coli* proficiency isolates, the accuracy levels of ribotyping (26.8%) and ARA (28.9%) were very similar overall. However, the level of accuracy for classifying human *E. coli* isolates was significantly better using ribotyping (62.5% ARCC) as compared to ARA (6.3%).

Reproducibility of Ribotyping Based on Proficiency Testing

Twenty "blind" proficiency isolates were tested in triplicate and identified to 1 of 7 possible source categories (Table 9). Of the 20 isolates tested, 2 isolates (10%) were correctly classified for all 3 reproducibility trials. Thirteen of 20 isolates (65%) were identified as the same source all 3 times, 2 of which was identified as the correct source. Five isolates agreed for 2 of 3 trials (66% > 100%), 2 of which were correctly classified. There was no significant difference in the results when the data was analyzed without including sewage isolates (data not shown).

C. Comparison of ARA and Ribotyping Results

Agreement Between ARA and Ribotyping

The agreement between the ARA and ribotyping methods was compared using the *E. coli* proficiency results (N=97). For both methods, only 6 of 97 isolates (6%) had identical and correct classifications: horse (N=3), human (N=1), cat (N=1) and dog (N=1). There was no significant difference in agreement between ARA and ribotyping when sewage was excluded as a category.

Reproducibility

The reproducibility testing of 20 isolates by ARA and ribotyping are summarized in Table 10. Ribotyping was superior to ARA in terms of reproducing the source of isolates. However due to the low accuracy level, in most trials the predicted source was not correctly identified.

Classification as Human and Sewage vs. Non-human Sources

The ability of ARA and ribotyping for classification E. coli and Enterococcus isolates as human and sewage vs. non-human group was compared. An ideal identification method is accurate, highly sensitive and specific. In this comparison human and sewage isolates were combined into one group while the cat, dog, horse and seagull isolates were pooled as the non-human group. The accuracy, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for both methods are presented in Table 11. Sensitivity is the fraction of human and sewage isolates that were correctly classified while specificity is the fraction of non-human isolates correctly identified. The PPV represents the percentage of human and sewage isolates identified as such. The NPV represents the percentage of non-human isolates identified as such. Accuracy is the sum all of correct classifications divided by the total number of isolates tested. The overall accuracy for classifying isolates as human and sewage vs. animal derived was 57% for E. coli and 60% for Enterococcus using ARA and 67% for *E. coli* using ribotyping. The ribotyping method had higher sensitivity, specificity, PPV, NPV and accuracy, as compared to ARA for correctly classifying E. coli isolates as human and sewage or non-human in origin. However, this result can be attributed to the fact that more human isolates were correctly classified by ribotyping (62.5%) as compared sewage isolates (7.1%). Similarly, the ARA method had the highest sensitivity (66%) for classifying Enterococcus as human and sewage, but this result reflects how well sewage isolates were classified (85.7%) as compared to 0% of the human isolates (Table 4). Ribotyping may be superior to ARA for discriminating *E. coli* isolates from humans as being "human and sewage". On the other hand, ARA may be more useful for classifying *Enterococcus* from sewage into this combined category.

5. Source Identification of Watershed Isolates

The watershed isolates collected during Phase II were not tested using ARA and ribotyping due to the low accuracy and reproducibility results obtained using proficiency isolates. The ARCC for *E. coli* was 29% for ARA and 27% for ribotyping as determined using 97 proficiency isolates. The probability of correctly classifying a given isolate by chance for ribotyping was one in seven (categories), or 14.3%. While the 27% correct classification rate for proficiency isolates by ribotyping is nearly twice the expected rate by chance, this is significantly lower than 75% or higher rate that was anticipated at the beginning of the study. In the case of ARA, the probability of correctly classifying a given isolate by chance was one in six (categories), or 16.7%. While the 29% correct classification rate of proficiency isolates by ARA also represents nearly twice the rate expected by chance, the accuracy is far below acceptable limits. The accuracy of ARA using *Enterococcus* was also low (ARCC, 46%). Both methods

showed poor reproducibility testing 20 isolates. Therefore, it was determined that for this study, the ARA and ribotyping methods would not be useful for accurately classifying *E. coli* and *Enterococcus* isolates as originating from dogs, cats, horses, seagulls, human or sewage effluent and influent.

6. Discussion

The basis for bacterial source tracking procedures is the assumption that there are species-specific strains of bacteria inhabiting the intestinal tract of humans and animals. Such species-specificity would need to extend over a wide geographic area to be useful for identifying sources of bacterial contamination. Given this assumption, for ARA, specificity would be based on differential exposure to antimicrobial agents, whereas for ribotyping or other DNA typing methods, it would be based on unknown host-specific factors. Currently, the major limitation to using these techniques is the lack of research supporting species-specificity. The validity of terms such as "resident" or "transient" bacterial strains used in previous publications is still questionable. It has not yet been well established that there are resident strains shared by a large percentage of an animal or human species or that there are fecal bacteria species specific to one species' intestinal tract.

Techniques commonly used to type isolates in epidemiological investigations of outbreaks such as pulsed-field gel electrophoresis (PFGE), ribotyping, serotyping and antimicrobial susceptibility testing are accepted by the scientific community due to extensive documentation in the literature. However, the efficiency of these techniques for identifying sources of fecal indicator bacteria in watersheds cannot be compared directly with epidemiological outbreak investigations. Whereas typing methods are used in epidemiological studies to identify unknown strains of organisms with a known source strain, in watershed source tracking studies they are used to identify patterns specific to bacteria in individual species. Further studies are also needed to determine bacterial population variation by host and geographic location to establish the potential use of source tracking techniques for watershed studies.

There are still a number of variables associated with source tracking methods that have not been established. These include the number of sources which must be discerned, the size of the known source database (library), organism used, number and types of host categories, number of isolates per known sources tested, type of statistical analysis used for data interpretation and variability due to geographic location. The libraries constructed in this study, particularly for ribotyping, were much larger and more specific as compared to libraries in previous studies (Hartel et al., 1999; Carson et al., 2001; Wiggins, 1996). Theoretically, large libraries consisting of thousands of isolates should result in high classification rates, as they are more representative of microbial populations

than small libraries. Small libraries of less than 300 isolates per source will result in high correct classification rates which can be shown to be an artifact by assessing the extent of random clustering and does not occur with large libraries (Whitlock et al., 2002). However, the optimal database size to achieve maximum accuracy of classification has yet to be determined.

The lack of standardized methods for database construction, analysis and interpretation of the results complicates the comparison of classification rates obtained from various studies. For example, in this study, 44% of E. coli 3477 library isolates and 48% of 3657 Enterococcus isolates were correctly classified into 6 source groups using ARA and discriminant analysis. These results are much lower than the 84% and 87% ARCCs reported by Wiggins (1999) and Hagedorn (1999) using the same method but with fecal streptococci as the bacterial indicator. However, a low ARCC (34%) was also reported in a similar study analyzing 319 E. coli isolates from 9 source categories (Guan et al., 2002). The differences in classification rates between various studies may be attributed to the types of antibiotics used, and changes in antibiotic resistance patterns of bacteria as a result of antibiotic treatment, dietary changes of the host as well as geographic differences. Guan attributed the 35% ARCC (as compared to the results obtained by Wiggins and Hagedorn) to three major factors: using E. coli rather than fecal streptococci or *Enterococcus* spp., types of antibiotics used and differences in diversities of the bacterial collections due to different sampling protocols, and sampling from a wider geographic area.

As for the ribotyping library results, 64% of 997 *E. coli* library isolates were correctly classified into 1 of 6 source categories, comparable to the 74% ARCC reported by Carson (2001) using 287 *E. coli* isolates and 8 source categories.

In this study, the ARCCs of ARA and ribotyping for proficiency isolates were much lower than the ARCCs of the respective libraries. Although the ARCC of the *E. coli* ARA library was 44%, only 29% of the proficiency isolates were correctly classified. Similarly, the ARCC of the *E. coli* proficiency ribotyping library was 64%, but only 27% of the proficiency isolates were correctly classified. In contrast, for *Enterococcus* the library and proficiency results were very similar. The ARCCs of the *Enterococcus* ARA library was 48% and 46% for proficiency isolates. One possible explanation for the differences in ARCC between the two indicators may be due to higher strain variability of *E. coli* versus *Enterococcus*, as well as a broader distribution of *E. coli* strains between different host groups

Accurate source determination can be difficult if the bacterial strains analyzed are very similar genetically. Closely related strains from different host species may be classified into the same category. On the other hand, identical strains with minute genetic differences may be misclassified into different categories (Parveen et al., 1999). Strain variation can also add difficulty to achieving highly reproducible results. The reasons for the low reproducibility results obtained in

this study using both methods have not yet been determined, but may be due in part to incomplete precision of the methodology.

To maximize the accuracy and representativeness of the libraries, *E. coli* and *Enterococcus* were isolated from human, animal and sewage samples within the vicinity of the San Juan Watershed. In this study, sewage influent and effluent were analyzed as source categories because public health officials are interested in the use of source tracking methods to determine sewage contamination. Interestingly, many of the *E. coli* and *Enterococcus* isolates from sewage were not classified as human-derived. However, because human isolates were obtained from hospital specimens, they may be different from human isolates may also affect classification rates.

Classification rates will also vary depending on the number of source categories used. Previous studies showed that ARCCs improved when source categories were combined (Wiggins et al., 1999; Guan et al., 2002). In this study, combining groups, such as dog and cat into a "pet" category increased the ARCC using ARA for the combined source category as compared to the individual category (data not shown). However, the disadvantage to pooling categories is the inability to track indicators to a specific animal group, although in some cases, discrimination to 3 categories (human, livestock and wildlife) may be sufficient for making management decisions. Therefore, the usefulness of ARA and ribotyping will also depend on the degree of species level discrimination necessary to provide sufficient information to watershed managers.

Bacterial typing methods may be more successful for tracking fecal sources in small, simple watersheds or geographic areas impacted by a few species (i.e., cow, wildlife and human) and with limited genetic variability. The San Juan Creek watershed is a large, complex watershed that encompasses highly urbanized and industrial areas, horse stables and rural regions. The lower end of SJC is a habitat for a variety of birds that can number in the hundreds. It is possible that the diversity of bacterial strains in this watershed is higher compared to those in other source tracking studies. The results of this study suggest that source tracking methods may not work as well for large watersheds impacted by numerous fecal sources as compared to confined areas impacted by fewer sources.

Most published source tracking studies were conducted using a single typing technique. In this study, two different typing methods were compared in terms of source classification using the same set of *E. coli* isolates. ARA classifies indicator organisms into pre-determined groups (host source categories) according to differences in antibiotic resistance patterns, whereas ribotyping is based on differences in genetic patterns. The results indicate that the methods were not comparable for classifying *E. coli* into the source categories selected for this study. Of the 97 *E. coli* isolates that were tested, only 6 were classified to the

same sources by both methods. The ARA and ribotyping methods also differed in their ability to classify human and sewage isolates. The ribotyping method was significantly better than ARA for classifying human *E. coli* isolates correctly as compared to ARA. However, ARA was superior for classifying *Enterococcus* and *E. coli* isolates from sewage. Thus, further investigation to assess the usefulness of ARA combined with ribotyping to improve source identification is needed.

7. Conclusions

- 1. In this study, the ARA and ribotyping methods did not demonstrate sufficient accuracy, discriminatory power, or reproducibility necessary to identify *E. coli* and *Enterococcus* isolates as originating from humans or animals, or to further discriminate isolates from specific groups such as dogs, cats, horses, seagulls, sewage and humans.
- 2. The accuracy levels of ARA and ribotyping should not be based solely on the internal accuracy of the library. Validation of source tracking methods should include accuracy testing using unknown isolates that are not part of the original database and are provided by an independent laboratory.
- 3. Source tracking methods are developing technologies that have not been rigorously tested. The theoretical basis for the techniques has not been well established. Additional investigation is needed to address critical factors such as the monitoring design, type of indicator bacteria used, size and representativeness of the database, number of fecal indicator sources, number of proficiency test samples, type of data analysis used to interpret source identification results, bacterial variation, and geographic differences.
- 4. ARA and ribotyping may be more successful in source tracking investigations of confined areas, with few potential sources of bacterial pollution (as demonstrated in previous studies). Further research is needed to assess the accuracy of these techniques before they are used on a routine basis to determine specific sources of pollution or remediation measures.
- 5. The accuracy of Bacterial Source Tracking (BST) methods as source assessment tools has not been well established, particularly in California watersheds. Watershed source identification studies should continue using intense environmental monitoring of fecal indicators to determine sources of pollution. To date, source tracking results obtained from BST methods should be interpreted cautiously to avoid implementing pollution prevention which may not be cost-effective or successful in watershed remediation efforts.

Table 1. E. coli and Enterococcus Isolates from Environmental WaterSamples Collected at San Juan Creek (SJC).

Station	Sample Site	Escherichia coli		Enterococcus spp.		
number		No. samples	No. isolates	No. samples	No. isolates	
SJ 02	Pacific Ocean at mouth of SJC	12	397	12	340	
SJ C2	East side of SJC at the mouth	12	423	12	367	
SJ 06	SJC below Pacific Coast Hwy	15	249	15	381	
SJ 10	SJC above Trabuco Creek	13	406	13	375	
SJ 25	Trabuco Creek	16	345	16	387	
	Total	68	1820	68	1850	

Table 2. Sources of *E. coli* and *Enterococcus* Isolates for Assemblage of ARA and Ribotyping Libraries.

	E	ischerichia co	Enterococ	cus spp.	
Source	No. Fecal Samples	No. Isolates for ARA Library	No. Isolates for Ribotyping Library	No. Fecal Samples	No. Isolates for ARA Library
Human	109	523	159	160	773
Cat	64	380	110	38	299
Dog	77	423	135	78	434
Seagull	157	693	157	148	682
Horse	92	497	159	81	400
Sewage (Influent)	53	480	155	54	553
Sewage (Effluent)	52	474	155	49	516
Totals	604	3470	1030	608	3657

	Number (%) of Isolates Classified As:									
E. coli										
Source ↓	Cat	Dog	Horse	Seagull	Human	Sewage	Total			
Cat	151 (39.7%)	104 (27.4%)	44 (11.6%)	16 (4.2%)	39 (10.3%)	26 (6.8%)	380			
Dog	71 (16.8%)	185 (43.7%)	39 (9.2%)	46 (10.9%)	57 (13.5%)	25 (5.9%)	423			
Horse	7 (1.4%)	65 (13.1%)	285 (57.3%)	48 (9.7%)	5 (1.0%)	87 (17.5%)	497			
Seagull	39 (5.6%)	143 (20.6%)	101 (14.6%)	276 (39.8%)	83 (12.0%)	51 (7.4%)	693			
Human	57 (10.7%)	99 (18.6%)	42 (7.9%)	79 (14.8%)	209 (39.3%)	46 (8.6%)	532			
Sewage	36 (3.8%)	91 (9.6%)	234 (24.6%)	99 (10.4%)	81 (8.5%)	411 (43.2%)	952			
Total	361	687	745	564	474	646	3477			
RCP ^ª	41.8%	26.9%	38.3%	48.9%	44.1%	63.6%				
						ARCC⁵	43.6%			
Enteroco	cci									
Source ↓	Cat	Dog	Horse	Seagull	Human	Sewage	Total			
Cat	104 (34.8%)	78 (26.1%)	13 (4.3%)	43 (14.4%)	38 (12.7%)	23 (7.7%)	299			
Dog	75 (17.3%)	168 (38.7%)	16 (3.7%)	90 (20.7%)	38 (8.8%)	47 (10.8%)	434			
Horse	9 (2.3%)	8 (2.0%)	302 (75.5%)	23 (5.8%)	14 (3.5%)	44 (11.0%)	400			
Seagull	61 (8.9%)	75 (11.0%)	32 (4.7%)	326 (47.8%)	105 (15.4%)	83 (12.2%)	682			
Human	98 (12.7%)	88 (11.4%)	38 (4.9%)	187 (24.2%)	272 (35.2%)	90 (11.6%)	773			
Sewage	58 (5.4%)	60 (5.6%)	181 (16.9%)	135 (12.6%)	61 (5.7%)	574 (53.7%)	1069			
Total	405	477	582	804	528	861	3657			
RCP	25.7%	35.2%	51.9%	40.5%	51.5%	66.7%				
						ARCC	47.7%			

Table 3. Internal Accuracy of ARA Library.Classification of Escherichia coli and Enterococci known isolates by source.

^aRate of Correct Prediction

^bAverage Rate of Correct Classification

	Number (%) of Isolates Classified As:									
E. coli										
Source ↓	Cat	Dog	Horse	Seagull	Human	Sewage	Total			
Cat	4 (28.6%)	3 (21.4%)	4 (28.6%)	2 (14.3%)	0 (0.0%)	1 (7.1)	14			
Dog	1 (7.1%)	1 (7.1%)	3 (21.4%)	2 (14.3%)	0 (0.0%)	7 (50.0%)	14			
Horse	0 (0.0%)	1 (9.1%)	8 (72.7%)	1 (9.1%)	0 (0.0%)	1 (9.1%)	11			
Seagull	0 (0.0%)	2 (14.3%)	4 (28.6%)	5 (35.7%)	0 (0.0%)	3 (21.4%)	14			
Human	1 (6.3%)	2 (12.5%)	5 (31.3%)	3 (18.8%)	1 (6.3%)	4 (25.0%)	16			
Sewage	2 (7.1%)	2 (7.1%)	11 (39.3%)	4 (14.3%)	0 (0.0%)	9 (32.1%)	28			
Total	8	11	35	17	1	25	97			
RCP ^ª	50.0%	9.1%	22.9%	29.4%	100%	36.0%				
						ARCC ^b	28.9%			
Enterococ	ci									
Source ↓	Cat	Dog	Horse	Seagull	Human	Sewage	Total			
Cat	3 (23.1%)	3 (23.1%)	1 (7.7%)	0 (0.0%)	2 (15.4%)	4 (30.8%)	13			
Dog	1 (7.1%)	5 (35.7%)	0 (0.0%)	1 (7.1%)	3 (21.4%)	4 (28.6%)	14			
Horse	1 (7.1%)	0 (0.0%)	11 (78.6%)	0 (0.0%)	1 (7.1%)	1 (7.1%)	14			
Seagull	0 (0.0%)	1 (7.1%)	1 (7.1%)	2 (14.3%)	2 (14.3%)	8 (57.1%)	14			
Human	7 (43.8%)	1 (6.3%)	3 (18.8%)	1 (6.3%)	0 (0.0%)	4 (25.0%)	16			
Sewage	0 (0.0%)	0 (0.0%)	1 (3.6%)	2 (7.1%)	1 (3.6%)	24 (85.7%)	28			
Total	12	10	17	6	9	45	99			
RCP	25.0%	50.0%	64.7%	33.3%	0%	53.3%				
						ARCC	45.5%			

Table 4. ARA Accuracy.Classification of *Escherichia coli* and Enterococci proficiency isolates by source.

^aRate of Correct Prediction

^bAverage Rate of Correct Classification

Table 5. ARA *E. coli* Reproducibility.Classification of *E. coli* proficiency isolates by source. 6-Category Analysis^a.

Isolate No.	True Source	Predicted Source Trial #1	Predicted Source Reproducibility Trials ^b (n=9)
1	САТ	SEAGULL	3C , 3D, 2G, 1HO
2	CAT	HORSE	1C , 4D, 4G
3	CAT	DOG	1C , 6D, 2 G
4	DOG	SEWAGE	9D
5	DOG	SEWAGE	1C, 4D , 3G, 1HO
6	DOG	HORSE	2C, 3HO, 4S
7	EFFLUENT	SEWAGE	7D, 2 HO
8	EFFLUENT	DOG	9D
9	HORSE	HORSE	2G, 7HO
10	HORSE	HORSE	9НО
11	HORSE	HORSE	1D, 4G, 4HO
12	HUMAN	CAT	8C, 1HU
13	HUMAN	SEWAGE	8G, 1S
14	HUMAN	SEWAGE	6D, 3G
15	INFLUENT	SEAGULL	6C, 2G, 1HO
16	INFLUENT	HORSE	2G, 4HO, 3S
17	INFLUENT	SEWAGE	7D, 2HO
18	SEAGULL	HORSE	1 G , 8HO
19	SEAGULL	SEAGULL	2C, 2D, 3G , 2HO
20	SEAGULL	SEAGULL	2D, 7 G

^aEffluent and Influent combined as "Sewage" category ^bC=cat, D=dog, G=gull, HO=horse, HU=human, S=sewage.

Table 6. ARA Enterococcus Reproducibility.Classification of Enterococcus proficiency isolates by source. Six-category analysis^a.

Isolate No.	True Source	Predicted Source Trial #1	Predicted Source Reproducibility Trials ^b (n=9)
1	САТ	SEWAGE	5C, 4S
2	DOG	DOG	3D, 5HO, 1S
3	DOG	SEAGULL	4G, 5S
4	DOG	HUMAN	4HU, 5S
5	EFFLUENT	SEWAGE	4D, 5S
6	EFFLUENT	SEWAGE	5G, 4S
7	EFFLUENT	SEWAGE	9S
8	HORSE	HUMAN	2D, 7S
9	HORSE	SEWAGE	1D, 2HO, 2HU, 1G, 3S
10	HORSE	САТ	1C, 6D, 1S
11	HUMAN	SEWAGE	9S
12	HUMAN	САТ	6C, 3D
13	HUMAN	SEWAGE	1C, 8HO
14	INFLUENT	SEWAGE	1D, 5HU, 3S
15	INFLUENT	SEWAGE	3G, 6S
16	INFLUENT	SEWAGE	6G, 3S
17	SEAGULL	HORSE	9HO
18	SEAGULL	SEWAGE	8C, 1D
19	SEAGULL	HUMAN	2HU, 7S

^aEffluent and Influent combined as "Sewage" category ^bC=cat, D=dog, G=gull, HO=horse, HU=human, S=sewage.

Table 7. Internal Accuracy of Ribotyping Library.

Classification of known *Escherichia coli* isolates by source.

	Number (%	6) Maximum	Similarity	Jackknife A	nalysis of <i>E</i>	<i>. coli</i> Riboty	ype Profiles	
Source ↓	Cat	Dog	Horse	Seagull	Human	Influent	Effluent	Total
Cat	80 (68.8%)	16 (20.0%)	1 (1.1%)	3 (2.2%)	11 (9.7%)	5 (4.3%)	0 (0.0%)	116
Dog	17 (13.8%)	83 (67.0%)	2 (1.8%)	8 (6.4%)	5 (3.7%)	7 (5.5%)	2 (1.8%)	124
Horse	2 (1.3%)	2 (1.3%)	131 (82.4%)	4 (2.5%)	0 (0.0%)	9 (5.7%)	11 (6.9%)	159
Seagull	4 (2.6%)	10 (6.4%)	7 (4.5%)	108 (68.8%)	13 (8.3%)	5 (3.2%)	10 (6.4%)	157
Human	12 (7.6%)	8 (5.0%)	1 (0.6%)	11 (6.9%)	120 (75.5%)	4 (2.5%)	3 (1.9%)	159
Influent	3 (2.2%)	13 (8.8%)	21 (14.7%)	12 (8.1%)	8 (5.2%)	69 (46.3%)	22 (14.7%)	148
Effluent	5 (4.0%)	16 (12.0%)	17 (12.8%)	12 (8.8%)	12 (8.8%)	27 (20.0%)	45 (33.6%)	134
Total	123	148	180	158	169	126	93	997
RCP ^ª	65.0%	56.1%	72.8%	68.4%	71.0%	54.8%	48.4%	
							ARCC [▶]	63.8%

^aRate of Correct Prediction

^bAverage Rate of Correct Classification

Table 8. Ribotyping Accuracy.

Number (%) of <i>E. coli</i> Isolates Assigned As:								
Source↓	Cat	Dog	Horse	Seagull	Human	Sewage	Total	
Cat	2 (14.3%)	5 (35.7%)	3 (21.4%)	3(21.4%)	0 (0.0%)	1 (7.1%)	14	
Dog	3 (21.4%)	5 (35.7%)	0 (0.0%)	4 (28.6%)	0 (0.0%)	2 (14.3%)	14	
Horse	2 (18.2%)	2 (18.2%)	4 (36.4%)	0 (0.0%)	0 (0.0%)	3 (27.3%)	11	
Seagull	2 (14.3%)	4 (28.6%)	1 (7.1%)	3 (21.4%)	3 (21.4%)	1 (7.1%)	14	
Human	1 (6.3%)	3 (18.8%)	0 (0.0%)	0 (0.0%)	10 (62.5%)	2 (12.5%)	16	
Sewage Influent	0 (0.0%)	2 (14.3%)	0 (0.0%)	4 (28.6%)	7 (50.0%)	1 (7.1%)	14	
Sewage Effluent	2 (14.3%)	4 (28.6%)	2 (14.3%)	4 (28.6%)	1 (7.1%)	1 (7.1%)	14	
Total	12	25	10	18	21	11	97	
RCP ^ª	16.7%	20.0%	40.0%	16.7%	47.6%	18.2%		
						ARCC [▶]	26.8%	

Classification of *Escherichia coli* proficiency isolates by source.

^aRate of Correct Prediction

^bAverage Rate of Correct Classification

Table 9. Ribotyping *E. coli* Reproducibility ^a.Classification of *E. coli* proficiency isolates by source.

Isolate No.	True Source	Predicted Source Trial #1	Predicted Source Trial #2	Predicted Source Trial #3
1	CAT	SEAGULL	САТ	CAT
2	САТ	DOG	DOG	EFFLUENT
3	CAT	HORSE	САТ	CAT
4	DOG	DOG	DOG	DOG
5	DOG	INFLUENT	INFLUENT	INFLUENT
6	DOG	EFFLUENT	EFFLUENT	EFFLUENT
7	EFFLUENT	HORSE	HORSE	HORSE
8	EFFLUENT	INFLUENT	INFLUENT	INFLUENT
8	HORSE	DOG	EFFLUENT	EFFLUENT
10	HORSE	EFFLUENT	EFFLUENT	EFFLUENT
11	HORSE	EFFLUENT	DOG	SEAGULL
12	HUMAN	HUMAN	САТ	CAT
13	HUMAN	INFLUENT	САТ	SEAGULL
14	HUMAN	INFLUENT	INFLUENT	INFLUENT
15	INFLUENT	SEAGULL	SEAGULL	SEAGULL
16	INFLUENT	INFLUENT	INFLUENT	INFLUENT
17	INFLUENT	HUMAN	HUMAN	HUMAN
18	SEAGULL	DOG	DOG	DOG
19	SEAGULL	САТ	САТ	САТ
20	SEAGULL	INFLUENT	INFLUENT	INFLUENT

^aBased Pearson's Coefficient Correlation (2.0% tolerance. Influent and effluent isolates were not used in the library data set for unknown identification).

Method Organism (No. trials)	No. isolates	No. isolates with 100% reproducibility ^a (No. correctly identified)	No. isolates with 66% > 100% reproducibility ^b (No. correctly identified)	No. isolates with < 66 % reproducibility ^b
ARA <i>E. coli</i> (N=9)	20	3 (1)	10 (2)	7
Ribotyping <i>E. coli</i> (N=3)	20	13 (2)	5 (2)	2
ARA Enterococcus (N=9)	19	3 (1)	8 (1)	8

 Table 10.
 Summary of ARA and Ribotyping Reproducibility.

^aNumber of isolates identified into the same category for all trials ^bNumber of isolates identified into the same category for at least 6 of 9 trials by ARA or 2 out of 3 trials by ribotyping

Table 11. Accuracy of ARA and Ribotyping for Classifying *E. coli* Isolates as Human and Sewage^a vs. Non-human^b (Animal-derived).

	ARA E. coli		Ribotyping <i>E. coli</i>		ARA Enterococcus	
			Source of	Isolates		
Predicted Source	Human and Sewage (N=44)	Non- human (N=53)	Human and Sewage (N=44)	Non- human (N=53)	Human and Sewage (N=44)	Non- human (N=55)
Human and Sewage	14	12	22	10	29	25
Non- human	30	41	22	43	15	30
Sensitivity	32% (14	4/44)	50% (22/44)		66% (29/44)	
Specificity	77% (4	1/53)	81% (4	3/53)	55% (3	60/55)
PPV ^c	54% (14/26)		69% (22/32)		54% (29/54)	
NPV^d	58% (4	1/71)	66% (4	3/65)	67% (3	60/45)
Accuracy	57% (5	5/97)	67% (6	5/97)	60% (5	9/99)

^aCombining human, sewage influent and effluent categories ^bCombining cat, dog, seagull and horse categories ^cPositive Predictive Value ^dNegative Predictive Value

	ARCC (%) (no. isolates identified correctly/total no. isolates)		
	ARA	Ribotyping	
<i>E. coli</i> Library	44% (1517/3477) ^a	64% (636/997) ^a	
<i>E. coli</i> Proficiency Panel	29% (28/97) ^a	27% (26/97) ^b	
Enterococcus Library	48% (1746/3657) ^ª	Not done	
Enterococcus Proficiency Panel	45% (45/99) ^a	Not done	

Table 12. Summary of average correct classification rates (ARCC) for ARAand ribotyping libraries and proficiency panels.

^a 6 category analysis: cat, dog, horse, seagull, human, sewage
 ^b 7 category analysis: cat, dog, horse, seagull, human, influent, effluent

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